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Highly active and recyclable immobilized multiple enzymes for one-pot enantioselective cascade reactions: Synthesis of (*R*)- and (*S*)-α-amino acids from racemic α-hydroxy acids

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ABSTRACT

Cascade biotransformation of racemic alcohols to enantiopure amines is highly wanted in pharmaceutical manufacturing but requires green and efficient solution. Here we developed the first immobilized enzyme-based approach for this transformation, being cleaner and more productive than whole-cells biocatalysis, and more economic and higher-yielding than using isolated enzymes. As demonstration, the conversion of racemic mandelic acids to high-value (*R*)- or (*S*)-phenylglycines was achieved by immobilized enantioselective alcohol oxidase, racemase, catalase, and (*S*)- or (*R*)-transaminase. Co-immobilization of the four enzymes on Ni-NTA functionalised carriers gave high enzyme loadings (192–195 mg enzyme/g carrier), loading efficiencies (97–98%), and free enzyme activities (107–113%). Immobilized enzymes transformed racemic mandelic acids to the corresponding four (*S*)-phenylglycines in 91–99% *ee* and 82–95% yields or five (*R*)-phenylglycines in 93–99% *ee* and 83–94% yields. These catalysts showed good recyclability, retaining 86–98% productivities in the fifth reaction cycles.

1. Introduction

Biocatalysis allows for the green and efficient production of high-value chemicals in an environmentally benign manner [1]. Moreover, biocatalysis enables one-pot cascade reactions to provide sustainable manufacturing process by avoiding the separation of reaction intermediates, minimizing the waste generation, and enhancing the productivity through less accumulation of unstable or toxic intermediates [2–11]. Currently cascade biotransformations are often performed by mixing multiple isolated enzymes [12,13]. The use of nine isolated enzymes were even demonstrated, transforming 2-ethynylglycerol to islatravir in a three-step cascade [14]. Cascade biotransformation can also be carried out by using whole-cells expressing multiple enzymes [15–26]. Up to 10 enzymes could be expressed in a single *E. coli* strain, and the resulting whole-cell biocatalyst was able to convert L-phenylalanine to α-amino acids in an eight-step cascade [25]. While whole-cell catalysed cascade reactions are less expensive, they might encounter side reactions due to the existence of other enzymes in the cells as well as mass transfer limitation caused by cell membrane. In comparison, isolated enzyme-based cascades are cleaner with less side reactions and more efficient with less mass transfer limitation. However, isolated enzymes are less stable, more costly, and difficult to be obtained in large amounts [27,28]. Alternatively, cascade biotransformation could be carried out with immobilized enzymes, which could achieve cleaner and more efficient reactions as well as more economic process via enzyme recycling and reuse [29–31]. Nevertheless, in comparison to whole-cell and isolated enzyme-based cascades, immobilized multiple (>3) enzymes-based cascade reactions have been less developed [32–44], possibly due to the difficulty in making all immobilized enzymes with high activity, stability, and recyclability. Thus, we are interested in tackling this challenge by immobilizing four enzymes for cascade reactions, in the view of developing clean, efficient, and economic process for the cascade conversion of racemic alcohols to enantiopure amines [9,10,45,46], a key green chemistry reaction in pharmaceutical manufacturing [47].

Enantiopure amines are important pharmaceutical intermediates and racemic alcohols are easily available starting materials. Cascade biotransformation of racemic alcohols could provide simple access to enantiopure amines. The cascade reaction could be achieved by non-enantioselective oxidation of a racemic alcohol and enantioselective amination of the ketone intermediate [18,19,46,48–52]. In ADH-AmDH cascade, two enantiocomplementary alcohol dehydrogenase (ADHs)

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were used to convert a racemic alcohol to a ketone due to the lack of non-enantioselective ADH, followed by enantioselective amine dehydrogenase (AmDH) catalysed amination to prepare an enantiopure amine [49,53–56]. Such cascades require $NAD(P)^+$ and NAD(P)H for two reaction steps, respectively, and the automatic recycling of the co-factors through the cascade reaction is elegant. But efficient cofactor recycling still remains challenging, either by the use of the isolated enzymes or whole cells [49,53,54]. The problem could be partially solved by using a mixture of whole cells expressing ADHs and NOX and immobilized AmDH and GDH to enable separate cofactor regeneration, achieving 1410 times recycling of NADH [55]. On the other hand, ADH-transaminase (TA) cascades require different type of cofactors for the two reaction steps, thus avoiding the complex cofactor recycling in ADH-AmDH cascades. ADH-TA cascade was demonstrated for the conversion of a racemic alcohol by using whole-cell catalysts containing a (*S*)-enantioselective ADH, a racemase, and a (*R*)-TA to catalyse the amination to a (*R*)-amine [26]. An engineered ambidextrous ADH and an (*S*)-enantioselective TA expressed in whole-cells enabled the production of (*S*)-amines from racemic alcohols [57]. In such a cascade system, the regeneration of NAD^+ for the alcohol oxidation inside of cells still needs improvement. Cofactor independent alcohol oxidase (AOx) was recently used for the alcohol oxidation in the cascade. Combining a racemase, an (*S*)-enantioselective-AOx, catalase, and an enantioselective TA inside the cells allowed for the high-yielding transformation of a racemic α-hydroxy alcohol, for instance, 4-hydroxymandelic acid to enantiopure (*S*)- and (*R*)-α-amino acid, for instance, 4-hydroxyphenylglycine, without the need of nicotinamide cofactors [58]. To further enhance the productivity of this racemase-AOx-catalase-TA cascade, we decided to explore the immobilization of the four enzymes for the catalysis. Here, we report the first immobilized enzyme-catalysed cascade conversion of substituted mandelic acids to substituted phenylglycines (Scheme 1). Co-immobilization of hydroxymandelate oxidase, mandelate racemase, catalase, and (*S*)- or (*R*)-enantioselective transaminase on Ni-NTA resins afforded active, stable, and recyclable catalysts; and the immobilized enzyme-based cascades enabled the high-yielding, highly enantioselective, and clean production of the valuable (*S*)- and (*R*)-phenylglycines from easily available racemic mandelic acids.

2. Experimental methods

2.1. General procedure for the enzyme immobilization to prepare Ni-HMC, Ni-HMCE and Ni-HMCD

Mixtures of hydroxymandelate oxidase (H), mandelate racemase (M), and catalase (C), with or without the branched chain amino acid aminotransferase (E) or D-phenylglycine aminotransferase (D), and HisPur Ni-NTA resin in 10 mL potassium phosphate (KP) buffer (50 mM, pH 8.0) were shaken at 4 °C and 250 rpm for 30 min, followed by the centrifugation at 1000 g for 2 min to obtain the solid immobilized catalysts. The enzyme concentrations in the supernatants were measured by Bradford assay to deduce the amounts of enzymes immobilized on Ni-NTA resin. Then, enzyme loading efficiencies were calculated as the ratio of the amounts of immobilized enzyme and the initial amounts of enzymes used for the immobilization. Representative examples were given below.

Ni-HMC1: By using the above described procedure, 2.0 mg H, 20 mg M, and 0.5 mg C mixed with 115 mg Ni-NTA resin yielded 137 mg of Ni-HMC1 with specific enzyme loading of 190 mg/g Ni-NTA and enzyme loading efficiency of 97%.

Ni-HMCE1: By using the above described procedure, 2.0 mg H, 20 mg M, 0.5 mg C, and 30 mg E mixed with 265 mg Ni-NTA resin afforded 316 mg Ni-HMCE1 with specific enzyme loading of 192 mg/g Ni-NTA and enzyme loading efficiency of 97%.

Ni-HMCD1: By using the above described procedure, 2.0 mg H, 20 mg M, 0.5 mg C, and 30 mg D mixed with 265 mg Ni-NTA resin gave 316 mg Ni-HMCD1 with specific enzyme loading of 192 mg/g Ni-NTA, and enzyme loading efficiency of 97%.

2.2. Biotransformation of racemic mandelic acid ***1a*** to phenylglyoxylic acid ***2a*** using immobilized catalyst Ni-HMC1 or the free enzymes

10 mL KP buffer (0.2 M, pH 8.0) containing 20 mM *rac*-**1a** and 7.31 mg/mL of Ni-HMC1 (corresponds to 1.15 mg enzymes/mL: 0.1 mg H/mL, 1.0 mg M/mL and 0.05 mg C/mL) or 1.15 mg/mL of the free enzymes at the same ratio were shaken at 30 °C and 250 rpm. 50 μL aliquots were taken out at different time points for reverse-phase HPLC analysis to quantify the product **2a**. The analytical samples were prepared by mixing 50 μL aliquot with 650 μL ultrapure water (containing 0.1% trifluoroacetic acid) and 300 μL acetonitrile (containing 2 mM benzyl alcohol as internal standard), followed by the centrifugation at 13,000 g for 10 min

2.3. General procedure for the biotransformation of racemic mandelic acids ***1a-h*** to phenylglyoxylic acids ***2a-h*** with the recycling of catalysts Ni-HMC

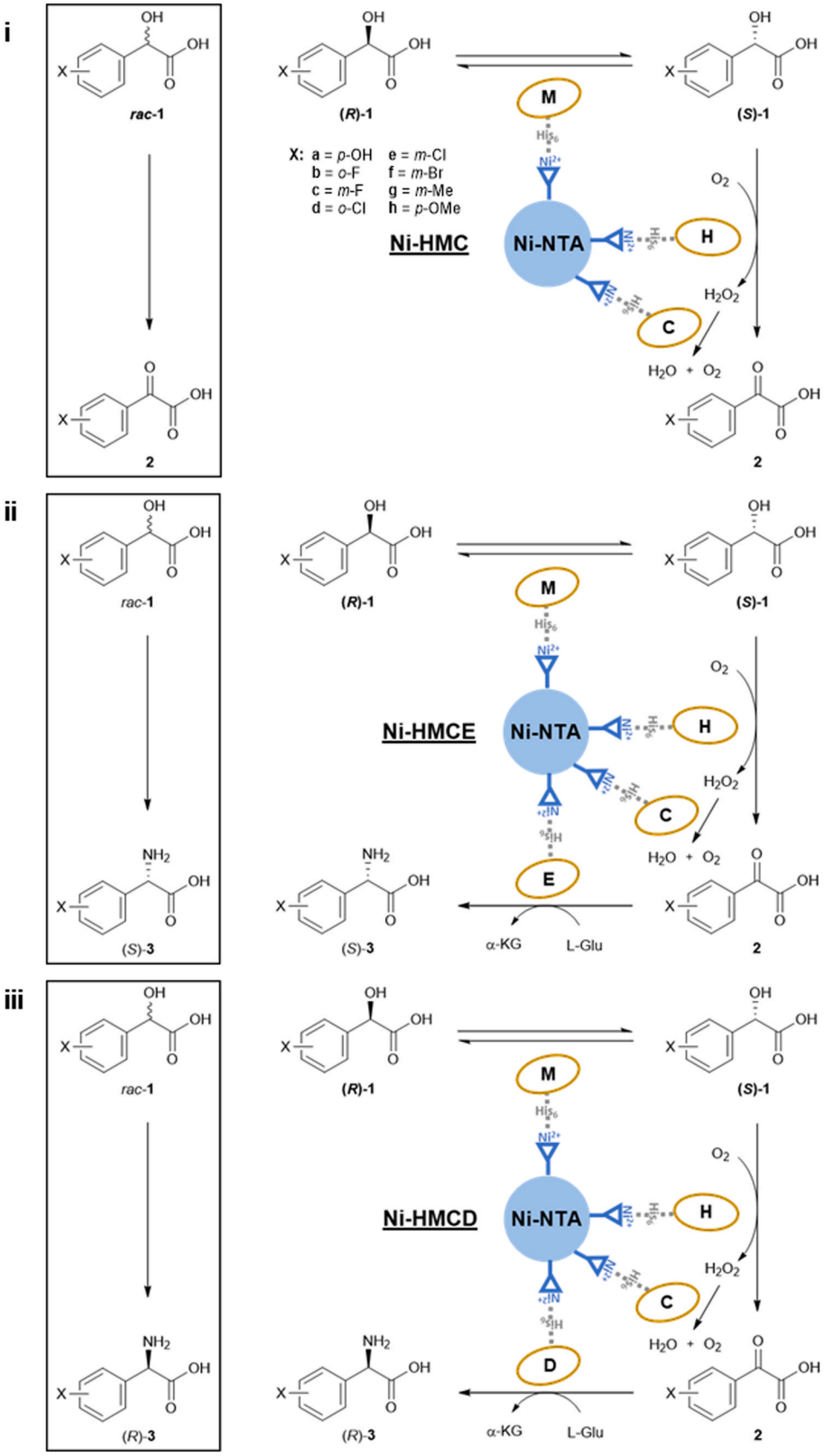
Mixtures of 5 – 20 mM *rac*-**1a-h** and 14.6 – 32.9 mg/mL of Ni-HMC1–4 in 5 mL KP buffer (0.2 M, pH 8.0) were shaken at 30 °C and 250 rpm for 4 h. Ni-HMC1–4 were separated from the reaction mixture by centrifugation at 1000 g for 2 min, and the supernatant was analysed on reverse-phase HPLC to quantify the product **2a-h**. The separated biocatalysts were washed with KP buffer (0.2 M, pH 8.0) twice, and added to 5 mL KP buffer (0.2 M, pH 8.0) containing the same concentrations of *rac*-**1a-h** as the first reaction cycle to perform the next reaction cycle. Detailed biotransformation conditions were given as follow: **2a-b**: 20 mM *rac*-**1a-b** with 14.6 mg/mL of Ni-HMC1; **2c**, **e**, **g**: 5 mM *rac*-**1c**, **e**, **g** with 15.7 mg/mL of Ni-HMC2; **2d**: 5 mM *rac*-**1d** with 32.9 mg/mL of Ni-HMC3; **2f**, **h**: 5 mM *rac*-**1f**, **h** with 18.9 mg/mL of Ni-HMC4.

2.4. Investigation of stability of Ni-HMC1 in KP buffer at 30 °C

13 shaking flasks containing 4.6 mL KP buffer (0.2 M, pH 8.0) with 36.6 mg Ni-HMC1 (corresponds to 5.75 mg enzymes: 0.5 mg H, 5.0 mg M and 0.25 mg C) were incubated at 30 °C and 250 rpm for 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40 or 50 days, respectively. After the specified days of incubation, 0.4 mL *rac*-**1a** was added to the reaction mixtures to the final concentration of 20 mM to perform the biotransformation to **2a** for 24 h. 50 μL aliquot was taken out for reverse-phase HPLC analysis of the conversions to **2a**. For comparison, 7 shaking flasks containing 4.6 mL KP buffer (0.2 M, pH 8.0) with 5.75 mg of the free enzymes at the same ratio were incubated at 30 °C and 250 rpm for 0, 1, 2, 3, 4, 5 or 10 days, respectively, and subjected to the biotransformation of *rac*-**1a** under the same condition as described for Ni-HMC1 to obtain the conversions to **2a**.

2.5. Biotransformation of racemic mandelic acid ***1a*** to (S)-phenylglycine ***3a*** using immobilized biocatalyst Ni-HMCE1 or the free enzymes

10 mL KP buffer (0.4 M, pH 8.0) containing 5 mM *rac*-**1a** and 32.9 mg/mL of Ni-HMCE1 (corresponds to 5.25 mg enzymes/mL: 0.2 mg H, 2.0 mg M, 0.05 mg C and 3.0 mg E) or 5.25 mg/mL of the free enzymes at the same ratio were shaken at 30 °C and 250 rpm. At 4 h, 0.1 mM PLP and 0.5 M L-Glutamate (L-Glu) were added to start the production of (*S*)-**3a** and the mixture was shaken for another 1 h. 50 μL aliquots were taken out at different time points for reverse-phase HPLC analysis to quantify the product (*S*)-**3a**. The analytical samples were prepared by mixing 50 μL aliquot with 850 μL ultrapure water (containing 0.1% trifluoroacetic acid) and 100 μL acetonitrile (containing 10 mM benzyl alcohol as internal standard), followed by the centrifugation at 13,000 g for 10 min.



Scheme 1. : i) One-pot production of phenylglyoxylic acids **2** from racemic mandelic acids **1** using immobilized biocatalyst Ni-HMC consisting of the enzymes H, M, and C on Ni-NTA resins. ii) One-pot production of phenylglycines (*S*)-**3** from phenylglyoxylic acid **2** using immobilized biocatalyst Ni-HMCE consisting of the enzymes H, M, C, and E on Ni-NTA resins. iii) One-pot production of phenylglycines (*R*)-**3** from phenylglyoxylic acid **2** using immobilized biocatalyst Ni-HMCD consisting of the enzymes H, M, C, and D on Ni-NTA resins. H: A80G/T159S/T162Q triple-mutant of hydroxymandelate oxidase from *S. coelicolor* A3(2); M: mandelate racemase from *P. putida*; C: catalase from *E. coli*; E: branched chain amino acid aminotransferase from *E. coli*; D: D-phenylglycine aminotransferase from *P. stutzeri* ST-201.

2.6. General procedure for the biotransformation of racemic mandelic acids **1a**, **b**, **d**, and **g** to (S)-phenylglycines **3a**, **b**, **d**, and **g** with the recycling of biocatalysts Ni-HMCE

Mixtures of 5 mM *rac*-**1a**, **b**, **d**, or **g** with Ni-HMCE1 (32.6 mg/mL), Ni-HMCE1 (32.6 mg/mL), Ni-HMCE2 (51.0 mg/mL) or Ni-HMCE3 (34.0 mg/mL), respectively, in 5 mL KP buffer (0.4 M, pH 8.0) were shaken at 30 °C and 250 rpm. At 4 h, 0.1 mM PLP and 0.5 M L-Glu were added to start the production of (*S*)-**3a**, **b**, **d**, or **g** and the mixtures were shaken for another 1 h. Catalysts Ni-HMCE were separated from the reaction mixtures by centrifugation at 1000 g for 2 min, and the supernatant was analysed on reverse-phase HPLC and chiral HPLC to quantify the product (*S*)-**3a**, **b**, **d**, or **g** and their *ee*, respectively. The separated catalysts were washed with KP buffer (0.4 M, pH 8.0) twice, and added to 5 mL KP buffer (0.4 M, pH 8.0) containing the same concentrations of *rac*-**1a**, **b**, **d**, or **g** as the first reaction cycle to perform the next reaction cycle.

2.7. Biotransformation of racemic mandelic acid **1a** to (R)-phenylglycine **3a** using immobilized biocatalyst Ni-HMCD1 or the free enzymes

10 mL KP buffer (0.4 M, pH 8.0) containing 5 mM *rac*-**1a** and 32.9 mg/mL of Ni-HMCD1 (corresponds to 5.25 mg enzymes/mL: 0.2 mg H, 2.0 mg M, 0.05 mg C and 3.0 mg D) or 5.25 mg/mL of the free enzymes at the same ratio were shaken at 30 °C and 250 rpm. At 4 h, 0.1 mM PLP and 1.5 M L-Glu were added to start the production of (*R*)-**3a** and the mixture was shaken for another 1 h. 50 μL aliquots were taken out at different time points for reverse-phase HPLC analysis to quantify the product (*R*)-**3a**. The analytical samples were prepared by mixing 50 μL aliquot with 850 μL ultrapure water (containing 0.1% trifluoroacetic acid) and 100 μL acetonitrile (containing 10 mM benzyl alcohol as internal standard), followed by the centrifugation at 13,000 g for 10 min.

2.8. General procedure for the biotransformation of racemic mandelic acids **1a**, **b**, **d**, **g**, and **h** to (R)-phenylglycines **3a**, **b**, **d**, **g**, and **h** with the recycling of biocatalysts Ni-HMCD

Mixtures of 5 mM *rac*-**1a**, **b**, **d**, **g**, and **h** with Ni-HMCD1 (32.6 mg/mL), Ni-HMCD1 (32.6 mg/mL), Ni-HMCD2 (51.0 mg/mL), Ni-HMCD3 (34.0 mg/mL) or Ni-HMCD4 (37.6 mg/mL), respectively, in 5 mL KP buffer (0.4 M, pH 8.0) were shaken at 30 °C and 250 rpm. At 4 h, 0.1 mM PLP and 1.5 M L-Glu were added to start the production of (*R*)-**3a**, **b**, **d**, **g**, and **h** and the mixtures were shaken for another 1 h. Catalysts Ni-HMCD were separated from the reaction mixture by centrifugation at 1000 g for 2 min, and the supernatant was analysed on reverse-phase HPLC and chiral HPLC to quantify the product (*R*)-**3a**, **b**, **d**, **g**, and **h** and their *ee*, respectively. The separated catalysts were washed with KP buffer (0.4 M, pH 8.0) twice, and added to 5 mL KP buffer (0.4 M, pH 8.0) containing the same concentrations of *rac*-**1a**, **b**, **d**, **g**, and **h** as the first reaction cycle to perform the next reaction cycle.

3. Results and discussion

3.1. Co-immobilization of hydroxymandelate oxidase, mandelate racemase, and catalase on Ni-NTA resin for the cascade biotransformation of ***rac*-α-hydroxy acid 1** to α-keto acid **2**

Various enzyme immobilization methods have been reported [29,31,59–61]. Among them, immobilization via bio-affinity interaction between histidine(his)-tag and metal(Ni, Co, Cu)–nitrilotriacetic acid (NTA) is promising due to the easy introduction of his-tag to the enzyme and the gentle immobilization condition [33,55,62–69]. Co-immobilization of hydroxymandelate oxidase (H), mandelate racemase (M), and catalase (C) on Ni-NTA resin was thus targeted for the efficient cascade biotransformation of *rac*-**1** to **2** under the optimized reaction conditions (30 °C and pH 8.0) [58]. Individual enzymes were first immobilized separately on Ni-NTA resin by shaking at 4 °C and 250 rpm for 30 min. Ni-NTA-hydroxymandelate oxidase (Ni-H) was prepared, with specific enzyme loading of 198 mg enzyme/g Ni-NTA, enzyme loading efficiency of 99%, specific activity of 3.8 U/mg and retained enzyme activity of 93% (Supplementary Table S1). Similarly, Ni-NTA-mandelate racemase (Ni-M) and Ni-NTA-catalase (Ni-C) were prepared with the specific enzyme loadings of 194 and 193 mg enzyme/g Ni-NTA, respectively, at the same enzyme loading efficiency of 97% (Supplementary Table S1). Ni-M retained 130% of the specific activity achieved with the free enzymes for the in racemization of (*R*)-**1a**. The enhanced specific activity of Ni-M is probably due to the enhanced enzyme stability as observed in other enzyme immobilization studies [67]. Ni-C retained 77% of the specific activity achieved with the free enzyme. Enzyme loading efficiencies of H, M, and C on Ni-NTA were high (97 – 99%) and suitable for co-immobilization on Ni-NTA under the same condition. Catalyst Ni-HMC1 was obtained by immobilization of 2 mg H, 20 mg M, and 0.5 mg C on 115 mg Ni-NTA resin, to give specific enzyme loading of 190 mg enzyme/g Ni-NTA, enzyme loading efficiency of 97%, and retained activity of 88% (Fig. 1a).

3.2. Cascade biotransformation of racemic mandelic acid **1a** to phenylglyoxylic acid **2a** using Ni-HMC1

Immobilized catalyst Ni-HMC1 was used for the cascade biotransformation of 20 mM *rac*-**1a** to **2a** with the catalyst loading of 7.3 mg/mL under the previously established optimized conditions [58] in potassium phosphate buffer (0.1 M, pH 8.0) at 30 °C and 250 rpm. The reaction progress was compared with the cascade biotransformation using purified enzymes of H (0.1 mg/mL), M (1.0 mg/mL), and C (0.05 mg/mL). As shown in Fig. 1b, conversion of *rac*-**1a** to **2a** using Ni-HMC1 rapidly increased to 68% at 2 h while the free enzymes achieved only 51% conversion in the same reaction period. Ni-HMC1 reached 92% conversion in 8 h and eventually reached 97% conversion at 24 h, which was more productive than the reaction using the free enzymes (86% conversion at 24 h). This suggests that the immobilization enhanced the productivity, possibly due to the enhanced enzyme stability. The enhancement in the activity of M after immobilization would also have contributed to the increase in overall productivity of the cascade.

3.3. Cascade biotransformation of racemic mandelic acid **1a** to phenylglyoxylic acid **2a** using Ni-HMC1 with catalyst recycling

To investigate the recyclability of immobilized catalyst Ni-HMC1, 14.6 mg/mL Ni-HMC1 was added to 20 mM *rac*-**1a** for the cascade biotransformation of *rac*-**1a** to **2a**. After 4 h, **2a** was produced with 99% conversion and Ni-HMC1 was separated from the reaction mixture by centrifugation for the next reaction cycle. The catalyst easily settled down to the bottom of the reaction mixture when the shaking was stopped, providing an efficient recovery of the catalyst after the reaction. The catalyst was reused for the new batch of the same reaction after each cycle of biotransformaton and catalyst recovery. Totally, five reaction cycles were performed, achieving 100%, 100%, 98% and 87% conversion relative to the conversion in the first cycle (Fig. 1c), affording an overall yield of 96% from the reaction of 100 mM *rac*-**1a** in the five reaction cycles. This demonstrates the success of recycling Ni-HMC for the target biotransformation.

3.4. Investigation of stability of Ni-HMC1

To determine if Ni-NTA resin could enhance the enzyme stability after immobilization, Ni-HMC1 was shaken in KP buffer (0.2 M, pH 8.0) at 30 ℃ and 250 rpm, which is the optimized reaction conditions [58], for 0–50 days. The sample was taken at different incubation time and subjected to biotransformation by adding *rac*-**1a**. The mixture of free enzymes H, M and C was used as control. As shown in Fig. 1d, Ni-HMC1

a)

H (mg)	M (mg)	C (mg)	Ni-NTA (mg)	Immobilized biocatalyst Ni-HMC1 (mg)[i]	Spec. enzyme loading (mg/g Ni-NTA)	Enzyme loading efficiency (%)	Specific activity (U/g Ni-HMC1)[ii]	Retained activity (%)[iii]
2.0	20	0.5	115	137	190	97	180	88

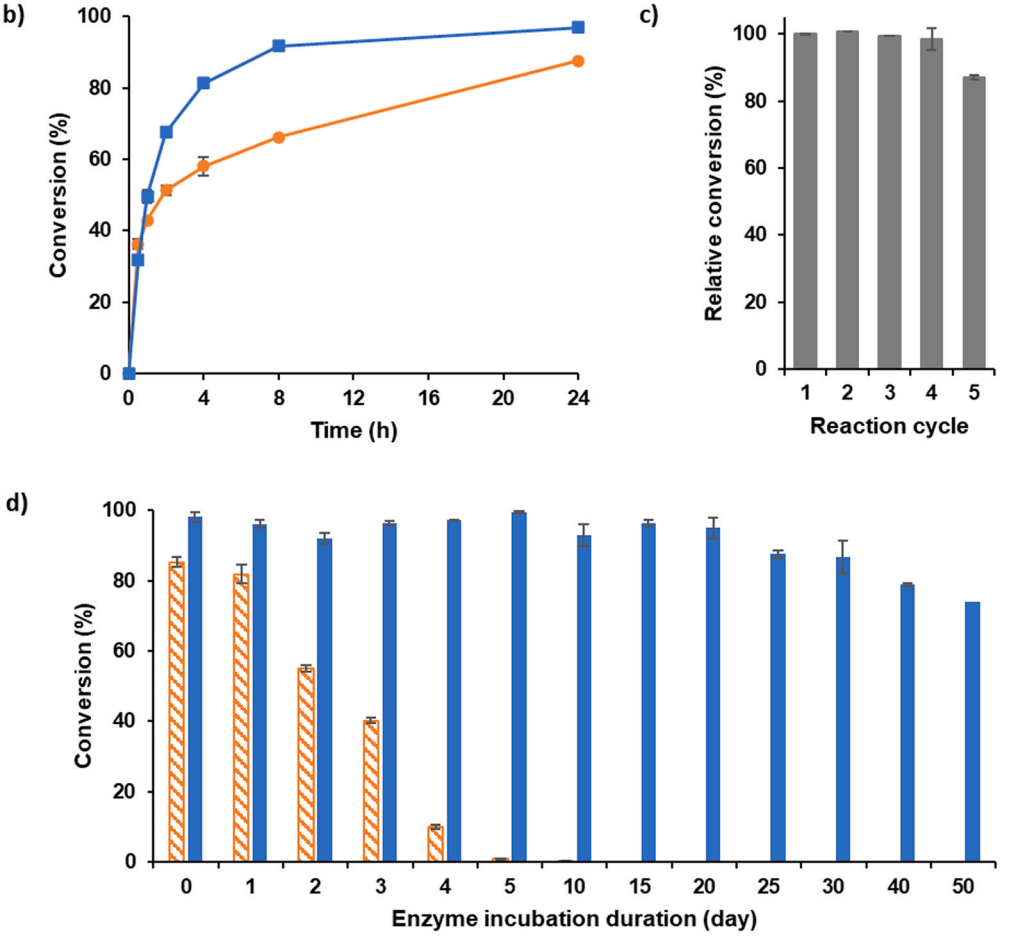


Fig. 1. a) Preparation of catalyst Ni-HMC1 by co-immobilization of the enzymes H, M and C on Ni-NTA resins. [i]Immobilization was performed in 15 mL KP buffer (0.05 M, pH 8.0) at 4 °C and 250 rpm for 30 min [ii]Specific activity was determined by reaction of 20 mM *rac*-**1a** with 7.3 mg Ni-HMC1/mL in KP buffer (0.2 M, pH 8.0) at 30 °C and 250 rpm for 30 min. U is defined as μmol **2a** produced per minute. [iii]Compared with the specific activity of the free enzymes. b) Reaction time courses of biotransformation of 20 mM *rac*-**1a** to **2a** using Ni-HMC1 (■) at 7.3 mg/mL (corresponds to 1.15 mg enzymes/mL: 0.1 mg H/mL, 1.0 mg M/mL and 0.05 mg C/mL) and free enzymes (●) at 1.15 mg/mL, respectively. c) Recycling of Ni-HMC1 for the biotransformation of *rac*-**1a** to **2a**. Reaction condition in each cycle: 20 mM *rac*-**1a**, 14.6 mg/mL Ni-HMC1 (or recycled), KP buffer (0.1 M, pH 8.0), 30 °C, 250 rpm, and 4 h. Relative conversion is based on the conversion at 4 h of the first reaction cycle. d) Stability of the immobilized biocatalyst Ni-HMC in KP buffer (0.1 M, pH 8.0) at 30 °C and 250 rpm. Ni-HMC1 (■) at 7.3 mg/mL (corresponds to 1.15 mg enzymes/mL: 0.1 mg H/mL, 1.0 mg M/mL and 0.05 mg C/mL). Free enzymes (□) at 1.15 mg/mL. After incubation for the specified number of days, 20 mM *rac*-**1a** was added to perform the biotransformation for 24 h.

retained > 90% conversion after 20 days, and 74% conversion after 50 days. In comparison, the free enzymes dropped the conversion rapidly from 85% to 40% after three days and almost zero after five days. These experiments clearly demonstrated that Ni-NTA resins could enhance the enzyme stability after immobilization.

3.5. *Immobilized catalysts Ni-HMC in cascade biotransformation of racemic mandelic acids **1b-h** to phenylglyoxylic acids **2b-h** with catalyst recycling*

To extend the application of Ni-HMC in the cascade biotransformation of the other substituted MAs to the corresponding PGAs, Ni-HMC1 was reacted with *rac*-**1b-h** to produce **2b-h**. The reaction of *rac*-**1b** for 4 h afforded **2b** with 99% conversion (Table 1). However, the conversions of *rac*-**1c-h** were rather poor (29 – 85%) even after 24 h. Analysis showed that **1c**, **1e**, **1f**, **1g**, and **1h** was remained in 47 – 91% and in 0.7 – 10% *ee* (*R*), indicating the inefficient (*S*)-enantioselective oxidation and efficient racemization in the related cascade reaction. Thus, Ni-HMC2 and Ni-HMC4 were prepared by increasing the amount of enzyme H to 5 and 10 mg, respectively, while not changing the other immobilization parameters. For the cascade conversion of *rac*-**1d** with Ni-HMC1, 50% substrate remained in 99% *ee* (*R*), which clearly suggested very poor racemization. Therefore, Ni-HMC3 was prepared by increasing the amount of enzyme M to 50 mg. The use of Ni-HMC2 for the reaction of *rac*-**1c**, **1e**, and **1g** and Ni-HMC4 for the reaction of *rac*-**1f** and **1h** gave the corresponding **2c, 2e-h** in 98 – 99% yields, respectively. The use of Ni-HMC3 enabled the conversion of *rac*-**1d** to **2d** in 97% after 4 h (Table 1).

After the completion of the first cycle of the reactions, Ni-HMC1–4 were recovered from the reaction mixture by centrifugation and then reused in the subsequent reaction cycles under the same reaction condition. As shown in Table 1, Ni-HMC1–4 demonstrated excellent stability and recyclability, retaining 89 – 97% of the original conversions in the fifth reaction cycles.

Table 1
Biotransformation of racemic mandelic acids **1a-h** to phenylglyoxylic acids **2a-h** using immobilized enzymes Ni-HMC as catalysts.

Subst.	X	Subst. conc. (mM)	Catalyst[a]	Catalyst loading (mg/mL)[b]	Immobil. individual enzyme loading[c]			Time (h)	Prod.	Conv. (%)	Relative conv. with the recycled catalyst[d]			
					H (mg/mL)	M (mg/mL)	C (mg/mL)				Cycle 2 (%)	Cycle 3 (%)	Cycle 4 (%)	Cycle 5 (%)
rac-**1a**	*p*-OH	20	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2a**	99	100	100	98	87
rac-**1b**	*o*-F	20	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2b**	99	98	97	96	93
rac-**1c**	*m*-F	5	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2c**	32	-	-	-	-
		5	Ni-HMC2	15.7	0.5	2.0	0.05	4	**2c**	99	100	100	99	98
rac-**1d**	*o*-Cl	5	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2d**	50	-	-	-	-
		5	Ni-HMC3	32.9	0.2	5.0	0.05	4	**2d**	97	99	94	91	89
rac-**1e**	*m*-Cl	5	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2e**	27	-	-	-	-
		5	Ni-HMC2	15.7	0.5	2.0	0.05	4	**2e**	99	100	97	96	92
rac-**1f**	*m*-Br	5	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2f**	13	-	-	-	-
		5	Ni-HMC2	15.7	0.5	2.0	0.05	4	**2f**	74	-	-	-	-
		5	Ni-HMC4	18.9	1.0	2.0	0.05	4	**2f**	99	100	99	99	97
rac-**1g**	*m*-Me	5	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2g**	53	-	-	-	-
		5	Ni-HMC2	15.7	0.5	2.0	0.05	4	**2g**	99	99	97	95	93
rac-**1h**	*p*-OMe	5	Ni-HMC1	14.6	0.2	2.0	0.05	4	**2h**	9.2	-	-	-	-
		5	Ni-HMC2	15.7	0.5	2.0	0.05	4	**2h**	53	-	-	-	-
		5	Ni-HMC4	18.9	1.0	2.0	0.05	4	**2h**	98	99	99	97	96

[a] Catalysts were prepared by immobilization of the enzymes H, M, and C on Ni-NTA in 15 mL KP buffer (0.05 M, pH 8.0) at 4 °C and 250 rpm for 30 min. Specific enzyme loading (mg enzyme/g carrier): 190 for Ni-HMC1, 194 for Ni-HMC2, 190 for Ni-HMC3, 193 for Ni-HMC4; enzyme loading efficiency (%): 97 for Ni-HMC1, 99 for Ni-HMC2, 96 for Ni-HMC3, 98 for Ni-HMC4. For details, refer to Supplementary Table 2.
[b] Refers to Ni-HMC catalysts in the biotransformation mixture.
[c] Refers to individual immobilized enzyme in the biotransformation mixture.
[d] Ni-HMC catalysts were recovered by centrifugation at 1000 g for 2 min and used for the next reaction cycle for 4 h under the same reaction condition as the first reaction cycle. The relative conversion is based on the conversion at 4 h of the first reaction cycle.

*3.6. Co-immobilization of hydroxymandelate oxidase, mandelate racemase, catalase, and (S)-transaminase on Ni-NTA resin for the cascade biotransformation of rac-α-hydroxy acid **1** to (S)-α-amino acid **3** with catalyst recycling*

Enzymes H (2 mg), M (20 mg), and C (0.5 mg) were co-immobilized with (*S*)-TA (E) (30 mg) on Ni-NTA resin (265 mg) to give Ni-HMCE1 for the cascade conversion of *rac*-**1a** to (*S*)-**3a**. Ni-HMCE1 prepared by using the same immobilization condition as described for Ni-HMC demonstrated specific enzyme loading of 192 mg enzyme/g Ni-NTA, enzyme loading efficiency of 97%, and retained activity of 107% (Fig. 2a). Under the optimized conditions (30 °C and pH 8.0) [58], Ni-HMCE1 was added to a mixture of 5 mM *rac*-**1a**, 0.5 M L-Glu, and 0.1 mM PLP to perform one-pot simultaneous cascade reactions. However, the conversion to (*S*)-**3a** was < 70% after 5 h reaction, with α-keto-acid substrate **2a** remained in the reaction mixture. This could possibly be due to the reverse reaction of the TA-catalysed second step reactions for totally 5 h. The reaction system was later on improved via one-pot concurrent manner as follows: Ni-HMCE1 and 5 mM *rac*-**1a** was first reacted for 4 h to give **2a**, followed by the addition of 0.5 M L-Glu and 0.1 mM PLP to perform amination reaction for 1 h. (*S*)-**3a** was produced with 98% *ee* and 82% yield (Fig. 2b). In comparison with the use of the free enzymes, Ni-HMCE1 gave slightly higher product yield. In comparison with one-pot simultaneous cascade, the one-pot concurrent cascade gave higher yield of (*S*)-**3a**, possibly due to the significantly reduced reaction time (1 h) for the reverse reaction of the second step.

Ni-HMCE1 was recovered from the reaction mixtures via centrifugation and then reused for the subsequent reaction cycles under the same reaction condition. Ni-HMCE1 showed impressive recyclability, achieving 94% conversion, relative to the first reaction cycles, in cycle 5 (Fig. 2c), and affording an overall yield of 79% (*S*)-**3a** from the cascade conversion of 25 mM *rac*-**1a**.

To compare the performance of immobilized enzymes with whole cells, the reaction of *rac*-**1a** to (*S*)-**3a** was selected as the representative example. While the whole-cell catalysis gave 0.02 mmol product/g cdw/h [58], the immobilized enzymes afforded 0.2 mmol product/g enzyme/h. Considering 1 g cells containing 0.5 g cell proteins with ca. 20% expression of the involved enzymes [58], the immobilized enzymes achieved at least the same productivity. Moreover, the immobilized enzymes could be recycled at least five times with high retained productivities, thus giving higher overall productivity than the use of whole cells. Furthermore, the immobilized enzymes gave much cleaner reaction and easier product recovery.

a)

H (mg)	M (mg)	C (mg)	E (mg)	Ni-NTA (mg)	Immobilized biocatalyst Ni-HMCE1 (mg)[i]	Spec. enzyme loading (mg/g Ni-NTA)	Enzyme loading efficiency (%)	Specific activity (U/g Ni-HMCE1)[ii]	Retained activity (%)[iii]
2.0	20	0.5	30	265	316	192	97	12.4	107

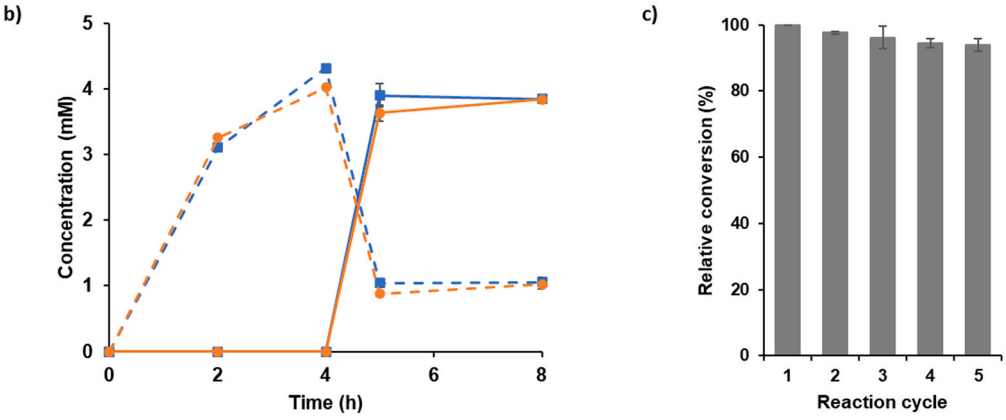


Fig. 2. a) Preparation of catalyst Ni-HMCE1 by co-immobilization of H, M, C, and E on Ni-NTA resins. [i]Immobilization was performed in 15 mL KP buffer (0.05 M, pH 8.0) at 4 °C and 250 rpm for 30 min [ii]Specific activity was determined by reaction of 5 mM *rac*-**1a** in the presence of 0.1 mM PLP and 0.5 M L-glutamate with 32.6 mg Ni-HMCE1/mL in KP buffer (0.4 M, pH 8.0) at 30 °C and 250 rpm for 1 h. U is defined as μmol of (*S*)-**3a** produced per minute. [iii]Compared with the specific activity of the free enzymes. b) Reaction time courses of cascade biotransformation of 5 mM *rac*-**1a** to (*S*)-**3a** using Ni-HMCE1 (■) at 32.9 mg/mL (corresponds to 5.25 mg enzymes/mL: 0.2 mg H, 2.0 mg M, 0.05 mg C and 3.0 mg E) and free enzymes (●) at 5.25 mg/mL, respectively. Solid line: (*S*)-**3a**; dashed line: **2a**. Reaction condition: At 0 h- 5 mM *rac*-**1a**, 32.9 mg/mL Ni-HMCE1, KP buffer (0.4 M, pH 8.0), 30 °C, 250 rpm; At 4 h- 0.1 mM PLP, 0.5 M L-glutamate and 1 h. c) Recycling of Ni-HMCE1 for the biotransformation of *rac*-**1** to (*S*)-**3** at the same condition described in b). Relative conversion is based on the conversion at 5 h of the first reaction cycle.

Table 2
Cascade biotransformation of racemic mandelic acids **1** to (*S*)-phenylglycines **3** using immobilized enzymes Ni-HMCE as catalysts, and to (*R*)-phenylglycines **3** using Ni-HMCD as catalysts.

Subst.	X	Subst. conc. (mM)	Catalyst[a]	Catalyst loading (mg/mL)[b]	Immobil. individual enzyme loading[c]					Time (h)[d]	Prod.	*ee* (%)	Conv. (%)	Relative conversion with recycled catalyst[e]			
					H (mg/mL)	M (mg/mL)	C (mg/mL)	E (mg/mL)	D (mg/mL)					Cycle 2 (%)	Cycle 3 (%)	Cycle 4 (%)	Cycle 5 (%)
rac-**1a**	*p*-OH	5	Ni-HMCE1	32.6	0.2	2.0	0.05	3.0	-	5	(*S*)-**3a**	98	82	98	96	94	94
rac-**1b**	*o*-F	5	Ni-HMCE1	32.6	0.2	2.0	0.05	3.0	-	5	(*S*)-**3b**	99	95	98	95	91	87
rac-**1d**	*o*-Cl	5	Ni-HMCE2	51.0	0.2	5.0	0.05	3.0	-	5	(*S*)-**3d**	93	85	95	95	95	95
rac-**1g**	*m*-Me	5	Ni-HMCE3	34.0	0.5	2.0	0.05	3.0	-	5	(*S*)-**3g**	91	91	99	97	97	97
rac-**1a**	*p*-OH	5	Ni-HMCD1	32.6	0.2	2.0	0.05	-	3.0	5	(*R*)-**3a**	99	83	100	100	99	96
rac-**1b**	*o*-F	5	Ni-HMCD1	32.6	0.2	2.0	0.05	-	3.0	5	(*R*)-**3b**	93	94	96	93	95	95
rac-**1d**	*o*-Cl	5	Ni-HMCD2	51.0	0.2	5.0	0.05	-	3.0	5	(*R*)-**3d**	93	86	95	93	93	92
rac-**1g**	*m*-Me	5	Ni-HMCD3	34.0	0.5	2.0	0.05	-	3.0	5	(*R*)-**3g**	97	83	99	99	96	92
rac-**1h**	*p*-OMe	5	Ni-HMCD4	37.6	1.0	2.0	0.05	-	3.0	5	(*R*)-**3h**	97	83	92	90	89	85

[a] Catalysts were prepared by immobilization of H, M and C together with E or D on Ni-NTA in 15 mL KP buffer (0.05 M, pH 8.0) at 4 °C and 250 rpm for 30 min. Specific enzyme loading (mg enzyme/g carrier): 192 for Ni-HMCE1, 193 for Ni-HMCE2, 195 for Ni-HMCE3, 192 for Ni-HMCD1, 193 for Ni-HMCD2, 193 for Ni-HMCD3, 192 for Ni-HMCD4; enzyme loading efficiency (%): 97 for Ni-HMCE1, 97 for Ni-HMCE2, 98 for Ni-HMCE3, 97 for Ni-HMCD1, 97 for Ni-HMCD2, 97 for Ni-HMCD3, 97 for Ni-HMCD4. For details, refer to Supplementary Table 2.

[b] Refers to Ni-HMCE and Ni-HMCD catalysts in the biotransformation mixture.

[c] Refers to individual immobilized enzyme in the biotransformation mixture.

[d] The corresponding reaction conditions are described in the legend of Fig. 2b and Fig. 3b, respectively.

[e] Ni-HMCE and Ni-HMCD catalysts were recovered by centrifugation at 1000 g for 2 min and used for the next reaction cycle for 5 h under the same reaction condition as the first reaction cycle. The relative conversion is based on the conversion at 5 h of the first reaction cycle.

3.7. Immobilized catalysts Ni-HMCE in cascade biotransformation of racemic mandelic acids **1b**, **d**, and **g** to (S)-phenylglycines **3b**, **d**, and **g** with catalyst recycling

Based on the results of Ni-HMC1–4 catalysed reaction of *rac*-α-hydroxy acid **1** to α-keto acid **2**, the appropriate amount of enzymes H, M, and C were co-immobilized with 30 mg (*S*)-TA (E) on Ni-NTA to give Ni-HMCE2 and Ni-HMCE3 (Table 2, entry 1–4), with 192 – 195 mg enzyme/g Ni-NTA and 97 – 98% enzyme loading efficiency (Supplementary Table S2).

The use of Ni-HMCE1 for the reaction of *rac*-**1b**, Ni-HMCE2 for the reaction of *rac*-**1d**, and Ni-HMCE3 for the reaction of *rac*-**1g** gave the corresponding (*S*)-**3b**, **3d**, and **3g** in 95%, 85% and 91% yields, respectively, with 91 – 99% *ee* (Table 2).

Ni-HMCE1–3 showed also as very good recyclability. The catalysts were recovered from the reaction mixture by centrifugation and then reused in the subsequent reaction cycles under the same reaction condition, retaining 87 – 97% of the original conversions in the fifth reaction cycles.

3.8. Co-immobilization of hydroxymandelate oxidase, mandelate racemase, catalase, and (R)-transaminase on Ni-NTA resin for the cascade biotransformation of rac-mandelic acid **1** to (R)-phenylglycine **3** with catalyst recycling

Enzymes H (2 mg), M (20 mg), and C (0.5 mg) were co-immobilized with (*R*)-TA (D) (30 mg) on Ni-NTA resin (265 mg) to give Ni-HMCD1 for the cascade conversion of *rac*-**1a** to (*R*)-**3a**. Ni-HMCD1 displayed specific enzyme loading of 192 mg enzyme/g Ni-NTA, enzyme loading efficiency of 97%, and retained activity of 113% (Fig. 3a). Ni-HMCD1 was used to transform *rac*-**1a** to (*R*)-**3a**. Under the optimized condition, Ni-HMCD1 and 5 mM *rac*-**1a** was first reacted for 4 h, followed by the addition of 1.5 M L-Glu and 0.1 mM PLP to perform amination reaction for 1 h. (*R*)-**3a** was produced with 99% *ee* and 83% yield (Fig. 3b). In comparison with the free enzymes, Ni-HMCD1 gave 10% higher product yield. The recycling experiment in reaction cycle 5 showed that Ni-HMCD1 achieved 96% conversion of the first reaction cycle (Fig. 3c), giving an overall yield of 82% of (*R*)-**3a** from the cascade reaction of 25 mM *rac*-**1a** in the five reaction cycles.

3.9. Immobilized catalysts Ni-HMCD in cascade biotransformation of racemic mandelic acids **1b**, **d**, **g**, and **h** to (R)-phenylglycines **3b**, **d**, **g**, and **h** with catalyst recycling

Similar to the preparation of Ni-HMCE1–3 in 3.7, the appropriate amounts of enzymes H, M, and C were co-immobilized with 30 mg D on Ni-NTA, affording Ni-HMCD2–4, with specific enzyme loading of 192 – 193 mg enzyme/g carrier and 97% enzyme loading efficiency (Supplementary Table S2). Ni-HMCD1-catalysed reaction of *rac*-**1b**, Ni-HMCD2-catalysed reaction of *rac*-**1g**, Ni-HMCD3-catalysed reaction of *rac*-**1g**, and Ni-HMCD4-catalysed reaction of *rac*-**1h** gave the corresponding (*R*)-**3b, 3d**, **3g** and **3h** in 93%, 93%, 97% and 97% yields, respectively, with 93 – 97% *ee* (Table 2). Ni-HMCD1–4 also showed high stability and recyclability, retaining 85 – 96% of the original conversions in the fifth reaction cycles. Currently, the biotransformation was performed in 5 – 10 mL scale. In the future, we will explore the scalability of the immobilized enzyme-catalysed biotransformation.

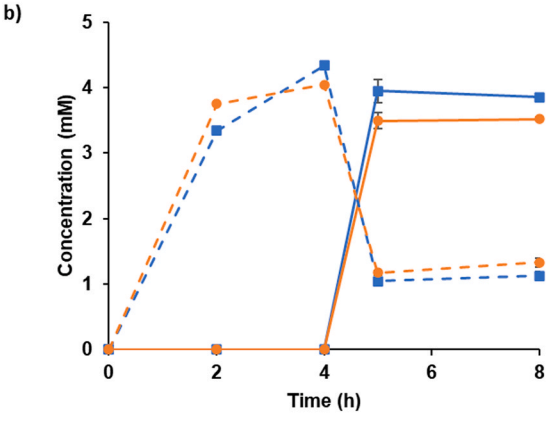
4. Conclusion

The first immobilized enzyme-catalysed cascade conversion of easily available racemic alcohols to enantiopure (*R*)- or (*S*)-amines has been successfully demonstrated, providing a useful alternative method for this type of key green chemistry reaction in pharmaceutical manufacturing. The cascade reactions were achieved in high yield and clean manner via alcohol oxidase (AOx)-transaminase (TA) without the requirement of nicotinamide cofactor by the use of immobilized enantioselective AOx, racemase, catalase, and (*S*)- or (*R*)-TA. Co-immobilization of the four enzymes on Ni-NTA functionalised carriers gave the catalysts (Ni-HMCE and Ni-HMCD) for cascade conversion of racemic α-hydroxy acids to (*S*)- or (*R*)-α-amino acids, with high specific enzyme loadings (192–195 mg enzyme/g carrier), enzyme loading

a)

H (mg)	M (mg)	C (mg)	D (mg)	Ni-NTA (mg)	Immobilized biocatalyst Ni-HMCD1 (mg)[i]	Spec. enzyme loading (mg/g Ni-NTA)	Enzyme loading efficiency (%)	Specific activity (U/g Ni-HMCD1)[ii]	Retained activity (%)[iii]
2.0	20	0.5	30	265	316	192	97	12.5	113

b)



c)

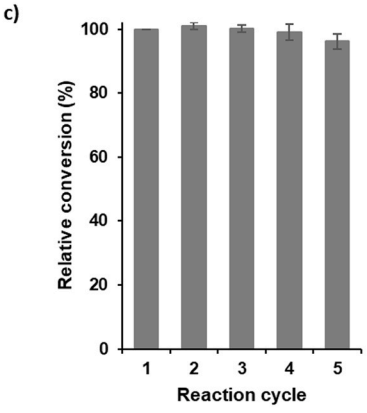


Fig. 3. a) Preparation of catalyst Ni-HMCD1 by co-immobilization of H, M, C, and D on Ni-NTA resins. [i]Immobilization was performed in 15 mL KP buffer (0.05 M, pH 8.0) at 4 °C and 250 rpm for 30 min [ii]Specific activity was determined by reaction of 5 mM *rac*-**1a** in the presence of 0.1 mM PLP and 1.5 M L-glutamate with 32.6 mg Ni-HMCD1/mL in KP buffer (0.4 M, pH 8.0) at 30 °C and 250 rpm for 1 h. U is defined as μmol of (*R*)-**3a** produced per minute. [iii]Compared with the specific activity of the free enzymes. b) Reaction time courses of cascade biotransformation of 5 mM *rac*-**1a** to (*R*)-**3a** using Ni-HMCD1 (■) at 32.9 mg/mL (corresponds to 5.25 mg enzymes/mL: 0.2 mg H, 2.0 mg M, 0.05 mg C and 3.0 mg D) and free enzymes (●) at 5.25 mg/mL, respectively. Solid line: (*S*)-**3a**; dashed line: **2a**. Reaction condition: At 0 h- 5 mM *rac*-**1a**, 32.9 mg/mL Ni-HMCD1, KP buffer (0.4 M, pH 8.0), 30 °C, 250 rpm; At 4 h- 0.1 mM PLP, 1.5 M L-glutamate and 1 h. c) Recycling of Ni-HMCD1 for the biotransformation of *rac*-**1** to (*R*)-**3** at the same condition described in b). Relative conversion is based on the conversion at 5 h of the first reaction cycle.

efficiencies (97–98%), and retained free enzyme activities (107–113%). By using the immobilized enzymes (Ni-HMCE and Ni-HMCD) to catalyse the cascade conversion of racemic mandelic acids, four (*S*)-phenylglycines were obtained in 91 – 99% *ee* and 82 – 95% yields, and five (*R*)-phenylglycines were afforded in 93 – 99% *ee* and 83 – 94% yields, respectively. The immobilized enzymes (Ni-HMCE and Ni-HMCD) showed good stability and recyclability, retaining 85 – 97% of the original conversions in the fifth reaction cycles. The developed cascades with the immobilized enzymes provided a cleaner and more productive alternative than the use of the whole-cell biocatalysts as well as an economic and higher yielding approach than the use of isolated enzymes. The established catalysis system enabled simple and practical access to (*R*)- and (*S*)-phenylglycines, which are useful and valuable intermediates for pharmaceutic manufacturing, from the easily available racemic mandelic acids.

CRediT authorship contribution statement

Do-Yun Jung: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Zhi Li:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zhi Li reports financial support was provided by GSK. Zhi Li reports financial support was provided by Singapore Economic Development Board.

Data Availability

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.apcatb.2023.123313.

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